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Fusion Competence of Interspecies Nuclear Transfer Embryos Reconstructed from Pedicle Periosteum Cell of Sika Deer (*Cervus nippon*) and Oocyte of Bovine

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ABSTRACT

The electrical fusion procedure used in Nuclear Transfer (NT) is one of the critical factors affecting the efficiency of animal cloning. The objective of this study was to compare the fusion competence of sika deer (*Cervus nippon*) and bovine interspecies nuclear transfer (iSCNT) in different electrical fusion parameters. The NT-embryos were obtained by transfer of the pedicle periosteum cell of deer at the 4th passage into the enucleated metaphase II (M II) bovine oocytes. As results shown, the percentage of couplets successfully fused at 2.4 kV cm^{-1} was the highest (57.2% vs 31.5, 41.0 and 45.7%, $p<0.05$). The percentage of couplets successfully and reconstructed embryos cleaved were the highest in 1 Direct Current (DC) pulse group but there were no significant difference among all groups ($p>0.05$). The percentage of couplets successfully fused in 10 μsec group (68.3%) was significantly higher than 40 μsec group (37.5%, $p<0.05$). The percentage of reconstructed embryos cleaved was 64.7% at 2 h group, it was higher than 3 h group (26.0%, $p<0.05$). These results suggested that electrical fusion procedure of 2.4 kV cm^{-1} EFS, 1 DC pulse, 10 μsec and 2 h after NT manipulation were feasible to iSCNT of sika deer-bovine.

Key words: iSCNT, electrical fusion, electrical field strengths, sika deer, bovine

INTRODUCTION

Since 1997, when scientists announced the birth of Dolly the sheep, the 1st cloned mammal (Wilmut *et al*., 1997), Somatic Cell Nuclear Transfer (SCNT) has been used to clone several mammalian species including cattle (Kato *et al*., 1998), mice (Wakayama and Yanagimachi, 1999), goats (Baguisi *et al*., 1999), pigs (Polejaeva *et al*., 2000), cats (Shin *et al*., 2002), rabbits (Chesne *et al*., 2002), horses (Galli *et al*., 2003), mules (Woods *et al*., 2003) and dogs (Lee *et al*., 2005). ISCNT is an invaluable tool for studying nucleous-cytoplasm interactions and play vital role in species preservation, whose oocytes are difficult to obtain (Kaedei *et al*., 2010), livestock propagation and therapeutic cloning (Abdullah *et al*., 2011). There were some animals such as asguar (Vogel, 2001), mouflon (Loi *et al*., 2001), African wildcat (Gomez *et al*., 2004), gray wolves (Oh *et al*., 2008) and other species born used iSCNT technique. Usually, in iSCNT, whole donor

cells, including the cytoplasm, are electro-fused into enucleated oocyte cytoplasts (Wilmut *et al*., 1997; Polejaeva *et al*., 2000; Karja *et al*., 2006; Daniel *et al*., 2008). As a direct and indirect consequence of human activities, only 2 subspecies, *Cervus nippon sinchuanicus* and*Cervus nippon kopschi*, currently subsist in the wild of China. However, a large population of *Cervus nippon hortulorum* and *Cervus nippon nippon* is raised in order to gain deer parts for Chinese traditional medicine (Wu *et al*., 2005). Because of the economic value raised, farmers slaughtered domestic sika deer haphazard in China, the population of sika deer declined sharply. The application of assisted reproduction technologies (Arts *et al*., 2013) such as iSCNT in sika deer breeding program becomes a limelight on today.

The present study is a preliminary effort to standardize the electric fusion protocol by applying different EFS, number of pulse, pulse length and time after Nuclear Transfer (NT) to increase fusion efficiency, using pedicle periosteum cell as donor cells and enucleated M II bovine oocytes as recipient cells and to find the feasible conditions for iSCNT of sika deer-bovine.

MATERIALS AND METHODS

Oocyte retrieval and *in vitro* maturation: Bovine ovaries were obtained from a local slaughter house and transported to the laboratory in sterile physiological saline supplemented with Penicillin (800 IU mL^{-1}) and Streptomycin (1000 IU mL^{-1}). Cumulus-oocyte-complexes (COCs) were collected from follicles 2-8 mm in diameter by aspiration using a 5 mL syringe with 16 gauge needle. The collected COCs were washed 5-8 times in PBS (GIBCO) droplets followed by washing 3 times in medium of *In vitro* Maturation (IVM) before cultured in 50 μL droplets of IVM medium overlaid with mineral oil under a humidified atmosphere of 5% CO_2 in air at 38.5°C for 22 h. IVM medium was based on M199 (GIBCO) supplemented with 0.33 mM sodium pyruvate, 10 IU mL^{-1} hCG, 15 IU mL^{-1} PMSG, 10% (v/v) fetal bovine serum (FBS, GIBCO).

Donor cell preparation: Sika deer pedicle periosteum cells were prepared as described previously (Li and Suttie, 2003). Cells were suspended with Dulbecco's Modified Eagle Medium (DMEM, GIBCO) containing 10% FBS and the suspension was centrifuged at 1,000 rpm for 5 min. The cell pellet was resuspended and cultured in DMEM supplemented with 10% FBS and antibiotics. For donor cells to be used in NT, bovine ear skin cells were cultured until they reached confluence. Before SCNT, cells were treated with 0.05% trypsin for single-cell isolation. After 4-9 passages, these cells were used as donor cells for iSCNT.

NT: For SCNT, COCs after IVM treatment were denuded in 0.3% hyaluronidase (SIGMA-ALDRICH). Only matured oocytes with the first polar body were subjected to a 10 min treatment of 7.5 μg mL^{-1} cytochalasin B (SIGMA-ALDRICH) prior to enucleation. The matured oocytes were enucleated using the squeezing technique. A cut was made on the zona pellucide above the first polar body and 20-30% of the cytoplasm beneath the first polar body was squeezed out using a glass needle. The squeezed out cytoplasm was subjected to 5 μg mL^{-1} Hoechst 33342 (SIGMA-ALDRICH) stain and viewed under the fluorescence microscope in order to confirm a positive enucleation.

Electrical fusion: After cultured 1 h, a single EF cell was injected into the perivitelline space of an enucleated oocyte. A sika deer pedicle periosteum cell was injected into an enucleated bovine oocyte. When the enucleated oocyte was cultured 1-3 h, the donor cell-recipient cytoplast couplets

were then fused in Zimmermann fusion medium with the parameter of 2.0-2.6 kV cm^{-1}, 1-3 Direct Current (DC) pulse and 10-40 μsec using the fusion machine ECM 2001 Electro Cell Manipulator (BTX, San Diego, CA). They were then placed in CR1aa medium containing 5% FBS for 1 h.

Activation: For the activation of reconstructed oocytes, the fused couplets were activated in 5 μM Ion (SIGMA-ALDRICH) for 5 min and followed with 2.0 mM DMAP (SIGMA-ALDRICH) for 3 h.

***In vitro* culture:** For the *in vitro* culture of reconstructed oocyte, the reconstructed oocytes were cultured in CR1aa containing 5% FBS under a humidified atmosphere of 5% CO_2 in air at 38.5°C. The first observation of embryo cleavage was made on day 2 of post-activation.

Statistical analyses: All data were expressed as the Mean±SEM and analyzed using IBM SPSS Statistics version 21.0. The differences among mean percentages of couplets successfully fused, dead embryos after fusion and reconstructed embryos cleaved were analyzed by one-way ANOVA followed by duncan multiple range test. Significance was determined at $p<0.05$.

RESULTS

Effects of electrical field strengths on success in fusion, dead embryos after fusion and cleavage rate of sika deer-bovine iSCNT embryos were shown in Table 1. The efficiency of fusion progressively increased from 31.5% at 2.0 kV cm^{-1} to 41.0 and 57.2% at 2.2 and 2.4 kV cm^{-} ,[1] respectively but decreased to 35.7 at 2.6 kV cm^{-1}. The percentage of couplets successfully fused at 2.4 kV cm^{-1} was higher than others ($p<0.05$). The percentage of dead embryos and reconstructed embryos cleaved had no significant difference among all groups ($p>0.05$), there were 18.5% vs. 30.7, 22.2 and 30.9%; 17.8% vs. 27.3, 37.4 and 22.9%, respectively.

Effects of number of pulse on success in fusion, dead embryos after fusion and cleavage rate of sika deer-bovine iSCNT embryos were shown in Table 2. From the Table 2 we knew the percentage of couplets successfully, reconstructed embryos cleaved and dead embryos had no

Table 1: Effects of electrical field strengths on success in fusion, dead embryos after fusion and cleavage rate of sika deer-bovine iSCNT embryos

Electrical field strengths‡ (kV cm^{-1})	No. of replicates	Percentage of couplets successfully fused (n)	Percentage of dead embryos after fusion (n)	Percentage of reconstructed embryos cleaved (n)
2.0	5	31.5±4.6^{b} (18/54)	30.7±9.3^{a} (16/54)	27.3±9.4^{a} (6/18)
2.2	5	41.0±5.2^{b} (22/54)	22.2±7.3^{a} (13/54)	37.4±11.1^{a} (7/22)
2.4	5	57.2±0.9^{a} (29/51)	18.5±7.8^{a} (11/51)	17.8±11.0^{a} (5/29)
2.6	5	35.7±7.2^{b} (15/40)	30.9±11.0^{a} (13/40)	22.9±15.7^{a} (3/15)

^{ab}Means with different superscripts in a column were significantly different ($p<0.05$). ‡Fused with 1 h after NT, 1 DC pulse and pulse length of 20 μsec

Table 2: Effects of No. of pulse on success in fusion, dead embryos after fusion and cleavage rate of sika deer-bovine iSCNT embryos

No. of DC pulse‡	No. of replicates	Percentage of couplets successfully fused (n)	Percentage of dead embryos after fusion (n)	Percentage of reconstructed embryos cleaved (n)
1	3	55.6±10.0^{a} (22/40)	14.3±14.3^{a} (6/40)	23.7±16.1^{a} (7/22)
2	3	37.4±4.5^{a} (15/40)	17.1±8.9^{a} (6/40)	16.7±16.7^{a} (2/15)
3	3	36.0±6.0^{a} (12/35)	17.4±8.7^{a} (5/35)	20.0±20.0^{a} (3/12)

^{ab}Means with different superscripts in a column were significantly different ($p<0.05$). ‡Fused with 1 h after NT, EFS of 2.4 kV cm^{-1} and pulse length of 20 μsec

Table 3: Effects of pulse length on success in fusion, dead embryos after fusion and cleavage rate of sika deer-bovine iSCNT embryos

Pulse length‡ (μsec)	No. of replicates	Percentage of couplets successfully fused (n)	Percentage of dead embryos after fusion (n)	Percentage of reconstructed embryos cleaved (n)
10	4	68.3±3.9^{a} (27/39)	12.1±7.2^{a} (4/39)	47.0±17.2^{a} (13/27)
20	4	52.9±10.0^{ab} (20/36)	26.3±7.6^{a} (8/36)	26.0±11.1^{ab} (5/20)
30	4	53.8±9.3^{ab} (21/38)	22.9±9.1^{a} (8/38)	6.3±6.3^{b} (1/21)
40	4	37.5±7.2^{b} (16/40)	37.3±15.4^{a} (13/40)	0^{b} (0/16)

^{ab}Means with different superscripts in a column were significantly different ($p<0.05$). ‡Fused with 1 h after NT, EFS of 2.4 kV cm^{-1} and 1 DC pulse

Table 4: Effects of time after NT on success in fusion, dead embryos after fusion and cleavage rate of sika deer-bovine iSCNT embryos

Time after NT‡ (h)	No. of replicate	Percentage of couplets successfully fused (n)	Percentage of dead embryos after fusion (n)	Percentage of reconstructed embryos cleaved (n)
1	4	48.2±7.2^{a} (19/40)	6.7±4.1^{a} (3/40)	54.3±13.5^{ab} (11/19)
2	4	62.7±12.8^{a} (28/45)	11.6±6.5^{a} (5/45)	64.7±13.7^{a} (17/28)
3	4	53.6±3.9^{a} (24/44)	13.9±2.9^{a} (6/44)	26.0±5.0^{b} (16/24)

^{ab}Means with different superscripts in a column were significantly different ($p<0.05$). ‡Fused with EFS of 2.4 kV cm^{-1}, 1 DC pulse and pulse length of 10 μsec

significant difference among all groups ($p>0.05$) but in 1 DC pulse group, the percentage of couplets successfully and reconstructed embryos cleaved was the highest (55.6 and 23.7%, respectively) and the percentage of dead embryos was the lowest (23.7%).

Effects of pulse length on success in fusion, dead embryos after fusion and cleavage rate of sika deer-bovine iSCNT embryos were shown in Table 3. Percentage of couplets successfully fused in 10 μsec group was the highest one and it was significantly higher than 40 μsec group (68.3 vs. 37.5%, $p<0.05$) but it had no significant difference to 20 μsec and 30 μsec groups (52.9 and 53.8%). Percentage of reconstructed embryos cleaved in 10 μsec group (47.0%) was significantly higher than 30 μsec group (6.3%) and 40 μsec (0%) group ($p>0.05$). The percentage of dead embryos after fusion were similar among all groups ($p>0.05$) but it was the lowest in 10 μsec group (12.1%).

Effects of time after NT on success in fusion, dead embryos after fusion and cleavage rate of sika deer-bovine iSCNT embryos were shown in Table 4. The percentage of reconstructed embryos cleaved was 64.7% at 2 h group, it was higher than 1 h (54.3%) and 3 h (26.0%) groups ($p<0.05$). The percentage of couplets successfully fused and dead embryos after fusion were similar among all groups ($p>0.05$) but the percentage of couplets successfully fused was highest in 1 h group.

DISCUSSION

Bovine oocytes had potentiality to support the somatic cells from different species to develop in iSCNT. Some people achieved success of iSCNT embryo production in several species, such as rhesus monkey-bovine (Kwon *et al*., 2011), cat-bovine (Thongphakdee *et al*., 2008; Imsoonthornruksa *et al*., 2011) and dog-bovine (Westhusin *et al*., 2001; Murakami *et al*., 2005). In this study, donor cell was from sika deer, it was not inter-genus with bovine. We obtained the percentage of couplets successfully fused 68.3% (Table 3) and the percentage of reconstructed embryos cleaved 64.7% (Table 4). It provided the evidence of the viewpoint above and showed that iSCNT of sika deer and bovine was not within the parameters of the genetic relationship between them. At the same time, we found some couplets of donor cell-cytoplast dead 4-5 h after fusion, the cytoplasts were condensed and it was different from the fragments in others researches. Whether it caused by the diversity from sika deer and bovine or not, it required do some in-depth study of the problem following this study.

Cell fusion was used generally for bovine NT, in which the donor cell was put into the perivitelline space between the zona pellucida and the cytoplasm of an enucleated oocyte and then both cytoplasms were fused by electrical stimulation (Shin *et al*., 2001). The pioneer yielded the fusion rate of 47-88% with this method in bovine SCNT (Kato *et al*., 1998). Then, Shin *et al*. (2001) obtained the percentage of couplets successfully fused as 42-53%, use the procedure, double DC pulses of I.75-I.85 kV cm^{-1} for 15 μsec in BTX Electro cell Manipulator 2001. With the same manipulator as us used, we yielded the percentages of couplets successfully fused as similar efficiency as above, they were 55.6-68.3% (Table 1-3), it showed that the method of cell fusion was feasible to sika deer-bovine iSCNT.

Zimmerman's mammalian cell fusion medium was used in our study, EFS and pulse length influenced the fusion efficiency significantly, as the percentages of couplets successfully fused was 57.2% at 2.4 kV cm^{-1} EFS, one DC pulse for 20 μsec (Table 1), 68.3% at 2.4 kV cm^{-1} EFS, one DC pulse for 10 μsec (Table 3). But there was no significant difference in percentages of couplets successfully fused, dead embryos after fusion and reconstructed embryos cleaved with different number of DC pulse (Table 2). It could be caused by the synergistic action of EFS, pulse length and DC pulse, should be investigated in future. Used the same fusion medium with us, in cat-bovine iSCNT, Thongphakdee *et al*. (2008) obtained the fusion rate of 73.7%, used the fusion procedure of one DC pulse of 25 V for 25 μsec with a needle-type electrode (Thongphakdee *et al*., 2008). In rhesus monkey-cow iSCNT, they (Kwon *et al*., 2011) used an Electro-Cell Fusion apparatus (NEPA GENE, Chiba, Japan) in conditions of 2 pulses of direct current of 34-38 V for 15 μsec duration with 0.26 M calcium-free mannitol and the fusion rate was 56.13% from the total injected oocytes. From all above researches, although the same recipient of oocytes used in different iSCNT, the similar fusion results were obtained with different fusion procedure. So, we can recognize that the efficiency of electrical fusion in iSCNT is influenced by EFS, number of pulse and pulse length together and these parameters depend on the type of cell fusion media and electrode in the same time.

After incubated NT-couplets for an additional 0.5 or 2 h before fusion with somatic cells, no significant difference in the rate of blastocysts per reconstructed embryo was observed between the 2 groups (Du *et al*., 2007). Differently in this research, there was no significant differences in percentage of couplets successfully fused among all groups (Table 4) but the percentage of reconstructed embryos cleaved in group 2 (64.7%) was significantly higher than group 3 (26.0%). It showed that NT-couplets incubated a certain period before fusion could influence the development of reconstructed embryos in SCNT, the reason maybe that the damage of cytoplasm caused by NT manipulation recovered in such procedure. In other research, the cultured time after fusion and before activation could influence the embryonic development of SCNT (Aston *et al*., 2006). It also proved that a certain period incubated in the interval of contiguous procedure in SCNT was important.

CONCLUSION

Based on the results from this experiment, fusion competence of sika-bovine iSCNT can be influenced by Electrical Field Strengths (EFS), pulse length and time after NT. Electrical fusion procedure of 2.4 kV cm^{-1} EFS, 1 DC pulse, 10 μsec and 2 h after NT manipulation were feasible to iSCNT of sika deer-bovine.

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